Title

Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance.

Journal

The Journal of experimental medicine, 162(1)

ISSN

0022-1007

Authors

Ildstad, ST
Wren, SM
Bluestone, JA
et al.

Publication Date

1985-07-01

DOI

10.1084/jem.162.1.231

Peer reviewed

CHARACTERIZATION OF MIXED ALLOGENEIC CHIMERAS

Immunocompetence, In Vitro Reactivity, and Genetic Specificity of Tolerance

By SUZANNE T. ILDSTAD, SHERRY M. WREN, JEFFREY A. BLUESTONE, SUSAN A. BARBIERI, and DAVID H. SACHS

From the Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

To date, solid organ and bone marrow transplantation between genetically disparate individuals has depended upon nonspecific immunosuppressive agents to control chronic rejection. Although these agents are vital to suppression of graft rejection, they have their own associated toxicities, including susceptibility to opportunistic infections, osteoporosis, impaired hepatic or renal function, and an increased incidence of neoplasms. Chronic rejection, in varying degrees of severity, often persists despite the use of these agents.

Numerous methods have been attempted to induce specific transplantation tolerance. Experimental animal models have included neonatal bone marrow inoculation (1) and whole body irradiation, or fractionated total lymphoid irradiation coincident with bone marrow transplantation and immediate or deferred solid organ transplantation in adult recipients (2–4). As a preparation for subsequent organ allografts, these methods have the advantage of induction of specific, rather than nonspecific tolerance. However, bone marrow transplantation is currently limited by graft-vs.-host disease (GVHD)[1] (5), and a level of immunoincompetence probably resulting from failure of appropriate immune cell interactions in the reconstituted host (6–8). We have attempted to overcome these limitations in the murine system by reconstituting the lethally irradiated host with T cell–depleted syngeneic plus allogeneic bone marrow components (9). Such animals were specifically tolerant to donor-type, full-thickness tail skin grafts, and exhibited stable hematopoietic chimerism. In addition, survival rates were excellent, with no evidence for GVHD or wasting.

We have presumed that such mixed reconstituted animals are superior to fully allogeneic chimeras because the presence of the syngeneic components overcomes the immunoincompetence that results from restriction of immune cell interactions in fully allogeneic chimeras, while the allogeneic elements provide conditioning for induction of specific tolerance. We have now examined in detail patterns of reconstitution, immunocompetence, and in vitro reactivity of such

[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; Con A, concanavalin A; FITC fluorescein isothiocyanate; GVHD, graft-vs.-host disease; MEM, minimum essential medium; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MST, median survival time; PBL, peripheral blood lymphocyte; PFC, plaque-forming cell; RAMB, rabbit anti-mouse brain; SRBC, sheep red blood cell.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/7/0231/14 $1.00 231
Volume 162 July 1985 231–244

mixed allogeneically reconstituted animals. In addition, we have extended this model to a major histocompatibility complex (MHC)-disparate, noncongenic model to assess the influence of minor antigens on induction and maintenance of tolerance to hematopoietic cells and allogeneic tissues. The mixed allogeneic system offers a model for investigation of mechanisms of induction and maintenance of transplantation tolerance in the adult animal, with potential application to the human clinical setting.

Materials and Methods

Animals. 6–8-wk-old male C57BL/10Sn (B10), B10.BR, B10.D2, and C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Animals were housed in a specific pathogen-free facility at the National Institutes of Health.

Mixed Allogeneic Reconstitutions (B10 + B10.D2 → B10). Mixed allogeneic chimeras were prepared by a modification of the methods previously described (10). Briefly, inbred B10 male recipients were lethally irradiated with 950 rad from a ^{137}Cs source. Using sterile technique, bone marrow was flushed from the femurs and tibias of B10, B10.D2, B10.BR, and C3H/HeJ male mice with Media 199 (Gibco, Grand Island, NY) containing 50 μl/ml of gentamicin (MEM) using a 23-gauge needle. The marrow was mechanically resuspended in MEM by gentle aspiration through a 19-gauge needle, and the suspension was sterilely filtered through nylon mesh. The cells were then centrifuged to a pellet at 1,200 rpm for 10 min, resuspended in MEM, and counted. Bone marrow was T cell–depleted by treatment with a 1:60 dilution of rabbit anti–mouse brain (RAMB) (at a ratio of 10^8 cells per milliliter of RAMB) sera at 4°C for 30 min, as previously described (9). The cells were subsequently washed and resuspended in sterile filtered guinea pig complement (Gibco) at a 1:3 dilution in MEM (10^8 cells/ml) at 37°C for 30 min. Cells were then washed twice (1,200 rpm for 10 min) and resuspended in MEM at the concentration appropriate to allow injection of 1 ml final volume per animal. Recipient animals were reconstituted 4–6 h after lethal irradiation via injection of cells into the lateral tail vein, using a 27 gauge needle. Mixed allogeneically reconstituted animals received 5×10^6 T cell–depleted syngeneic bone marrow cells and 1.5×10^7 T cell–depleted allogeneic bone marrow cells unless otherwise specified. Such animals will be referred to as mixed allogeneic chimeras, mixed allogeneically reconstituted animals, or B10 + B10.D2 → B10. Fully allogeneically reconstituted mice received 1.5×10^7 T cell–depleted allogeneic bone marrow cells (B10.D2 → B10). Syngeneically reconstituted controls received 5×10^6 T cell–depleted syngeneic bone marrow cells (B10 → B10). Radiation controls were performed simultaneously to confirm adequacy of the lethal radiation dose.

Assays for Chimerism. Chimerism of peripheral blood lymphocytes (PBL) was assayed using a trypan blue exclusion microcytotoxicity test to determine the percentage of lymphocytes bearing host- or donor-type H-2 surface markers as previously described (11). Briefly, peripheral blood was collected into heparinized plastic serum vials. After thorough mixing, the suspension was diluted with 100 μl of MEM plus 0.1% gelatin, and layered over a cold Ficoll-Hypaque gradient (Bionetics Laboratory Products, Charleston, SC). After centrifugation for 90 s in a Beckman microfuge, the lymphocyte layer was aspirated from the saline-Ficoll interface, and washed with MEM plus 0.1% gelatin. Red blood cells were then ACK-lysed, and the remaining cells were washed and used in a trypan blue microcytotoxicity assay.

Skin Grafting. Skin grafting was performed by a modification of the method of Billingham (12). Full-thickness tail skin grafts were harvested from the tails of B10.D2, B10.BR, C3H/HeJ, and B10 mice. Mice were anesthetized with tribromoethanol injected intraperitoneally (13), and full-thickness defects were created surgically in the lateral thoracic wall. We were careful to preserve the panniculus carnosum. The grafts were covered with a double layer of vaseline gauze and a plaster cast. If more than one skin graft was placed on an animal, each defect for graft placement was separated by a 3-mm

skin bridge. Casts were removed on the eighth day. Grafts were scored daily for percent rejection, and rejection was considered complete when no residual viable graft was evident. Graft survivals were calculated by the life-table method (20), and the median survival time (MST) was derived from the time point at which 50% of grafts were surviving.

Mixed Lymphocyte Reactions (MLR). MLR were performed as previously described (14). Briefly, splenocytes were ACK-lysed, washed, and reconstituted in EHAA (NIH Media Unit, Bethesda, MD) supplemented with 0.75% fresh normal mouse serum, 0.09 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.05 mM 2-mercaptoethanol. 4×10^5 responders were cultured with 4×10^5 stimulators (2,000-rad irradiated cells) and varying numbers of cocultured chimeric cells or normal control cells in a total of 200 or 250 μl of media. Cultures were incubated at 37°C, 5% CO_2 for 4 d, pulsed on the fourth day with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA) and harvested on the fifth day with an automated harvester (Mash II; Microbiological Associates, Bethesda, MD).

Cell-mediated Lympholysis (CML). CML assays were performed using a modification of techniques previously described (14, 15). Briefly, RPMI 1640 medium (Gibco) was supplemented as above, except that 10% fetal calf serum (Gibco) was used in place of normal mouse serum. 4×10^6 responders were cocultured with 4×10^6 stimulators (2,000-rad irradiated cells) and 2×10^6 or 4×10^6 chimeric putative suppressor cells or normal control cells in 2 or 2.5 ml of medium at 37°C for 5 d. Mouse target blasts were stimulated with concanavalin A (Con A) (Miles-Yeda Laboratories, Inc., Israel) for 2 d. In some experiments, tumor targets were used. After 5 d, responders were harvested, counted, and resuspended at appropriate effector/target ratios with 10^4 ^{51}Cr-labeled blasts as targets. After 4.5 h, supernatants were harvested with the Titertek system, and specific lysis was calculated as follows: (experimental release − spontaneous release) × 100/(maximal [HCl] release − machine background). Spontaneous release was <25% of maximum release unless otherwise indicated.

Cellular depletions were performed on the chimeric cells to eliminate H-2^b- or H-2^d-bearing cells. Briefly, chimeric cells were incubated with B10 anti-B10.D2 sera or B10.D2 anti-B10 sera for 15 min at 37°C and 5% CO_2 (5×10^6 cells per milliliter of antibody at 1:20 dilution). Cells were washed and resuspended in screened, sterilely filtered rabbit complement (5×10^6 cells per milliliter of complement at 1:8 dilution in MEM) for 30 min at 37°C. The suspension was then diluted with MEM and layered over warm Lympholyte M (Accurate Chemical Scientific Comp., Westbury, NY) in 50 ml conical tubes, and washed at 1,200 rpm for 25 min to separate viable from nonviable cells. The buffy coat was aspirated and washed twice in MEM (1,200 rpm for 10 min). The cells were then resuspended in media for CML, counted, and the cell density adjusted to the appropriate concentration. Adequacy of depletions was determined by fluorescence-activated cell sorter using biotinylinated, protein-purified anti-H-2^b and anti-H-2^d monoclonal antibodies and fluorescein isothiocyanate (FITC)-avidin fluorescent stain as previously described (9, 16).

Plaque-forming Cell (PFC) Assays. Sheep red blood cell (SRBC) PFC assays were performed as previously described (17). Briefly, animals were immunized intravenously with 0.2 ml of a 1% sheep red blood cell suspension on day 0. Partial splenectomies were performed on day 5, and titrated numbers of spleen cells were assayed in triplicate for PFC responses. Values are expressed as plaques per 10^6 spleen cells. All experiments reported were recorded by an observer who had no knowledge of the identity of a given plate.

Results

Survival of Mice Reconstituted with Fully Allogeneic (B10.D2 → B10), Mixed Allogeneic (B10 + B10.D2 → B10), and Syngeneic (B10 → B10) Bone Marrow Inocula. Survival of the mixed allogeneically reconstituted mice was excellent. Animals showed no evidence for GVHD or wasting. However, in our special pathogen-free facility, long-term survival of our fully allogeneic chimeras was

also excellent, and not significantly different from that for syngeneically (B10 → B10) reconstituted recipients (Fig. 1). To further assess in vivo immunocompetence by means of survival characteristics, age-matched animals from each of the following groups were moved from our special facility to a conventional facility 6 mo after lethal irradiation and bone marrow reconstitution: (*a*) syngeneically reconstituted animals (B10 → B10); (*b*) fully allogeneic chimeras (B10.D2 → B10); and (*c*) mixed allogeneic chimeras (B10 + B10.D2 → B10) (Fig. 2). 50-d survival after relocation was 40% for fully allogeneic chimeras, 80% for mixed allogeneic chimeras, and 83% for syngeneically reconstituted mice. Three representative mice that died were sent for serologic testing and animal autopsy, and were found to have died from endemic viral infections, including mouse

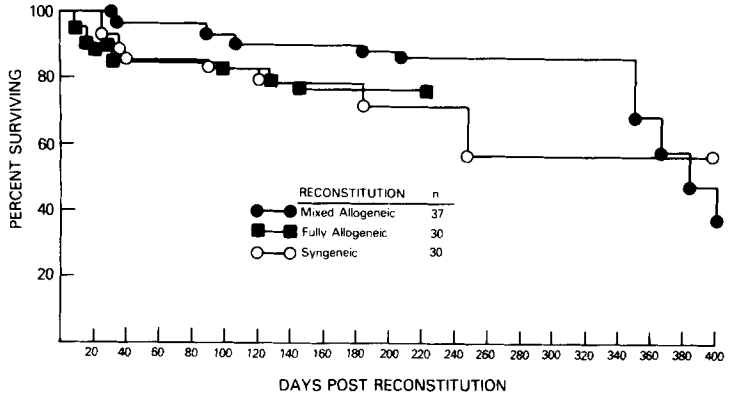

FIGURE 1. Survival of mixed allogeneic chimeras (B10 + B10.D2 → B10), fully allogeneic chimeras (B10.D2 → B10) and syngeneically reconstituted mice (B10 → B10) in our specific pathogen-free facility, as calculated by the life table method. Minimum follow-up was 79 d.

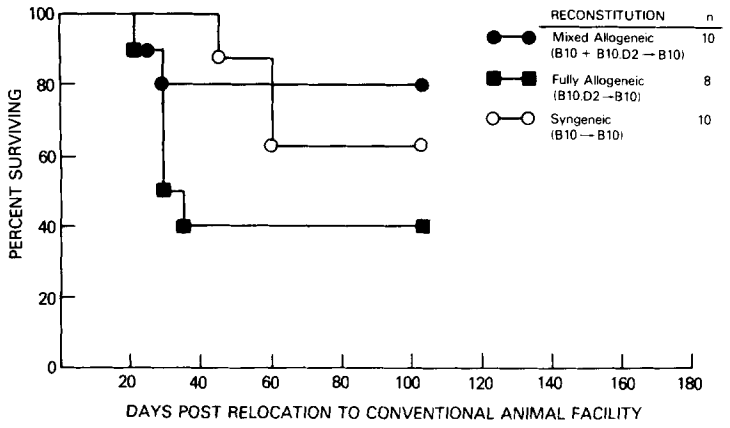

FIGURE 2. Survival of mice reconstituted with mixed allogeneic (B10 + B10.D2 → B10), fully allogeneic (B10.D2 → B10), or syngeneic (B10 → B10) bone marrow inoculum in a conventional facility. Mice were moved to a conventional facility 6 mo after lethal irradiation and reconstitution; before this they had been housed in a specific pathogen-free facility. Survivals were calculated by the life table method. Animals were followed for a minimum of 90 d postrelocation.

hepatitis virus and Sendai virus. In addition, *Citrobacter freundii* was isolated from the intestines of those animals.

Immunocompetence as Assessed by PFC Responses and Hemagglutination Assay. In an attempt to further assess in vivo immunocompetence of the mixed allogeneic chimeras (B10 + B10.D2 → B10), animals were subjected to intravenous immunization with SRBC suspensions, and their ability to form primary PFC responses quantitated. Mixed allogeneically reconstituted animals were found to be immunocompetent, with PFC responses similar to those of syngeneically reconstituted (B10 → B10) mice. As seen in Fig. 3, fully allogeneic chimeras B10.D2 → B10) responded less well. These data suggest that the mixed allogeneic chimeras are able to recognize foreign antigens in the context of self.

Typing of Mixed Allogeneic Chimeras (B10 + B10.D2 → B10); Time Course of

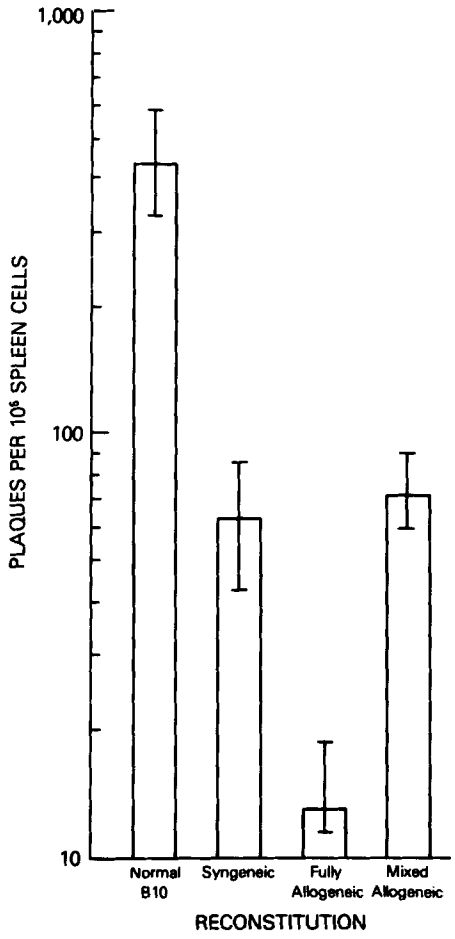

FIGURE 3. Immunocompetence as assessed by primary PFC responses to in vivo immunization with SRBC. Responses are expressed as the geometric mean of plaques per 10^6 spleen cells ⨲ SEM. Syngeneic (B10 → B10) (n = 9), mixed allogeneic chimeras (B10 + B10.D2 → B10) (n = 10), and fully allogeneic chimeras (B10.D2 → B10) (n = 8) were tested. Normal (control) B10 (n = 5).

Hematopoietic Repopulation. PBL typing of the mixed allogeneically reconstituted animals by trypan blue exclusion microcytotoxicity assay revealed variable percentages of donor-type lymphoid cells. Percentages of allogeneic lymphoid elements ranged from 5% to 95% in peripheral blood. However, in all animals, donor-type elements were identified. The time course of reconstitution of five fully allogeneic and five mixed allogeneic chimeras was followed serially (Table I). Although the absolute percentages of donor-type lymphoid elements was seen to fluctuate over time, the animals all remained chimeric.

Specific Tolerance to Donor-type Lymphoid Elements as Evidenced by MLR. As seen in a representative one-way MLR (Table II), mixed allogeneic chimeric responding cells (B10 + B10.D2 → B10) were specifically unreactive to donor (B10.D2) and host-type (B10) stimulator cells, while the response to third-party (B10.BR) cells was intact.

The response of normal control B10 lymphocytes against B10.D2 splenocytes in coculture with chimeric cells has revealed no evidence for specific or nonspecific suppression from 3 wk to >1 yr after mixed allogeneic reconstitution. Experiments included titration of increasing numbers of cocultured chimeric cells with 4×10^5 normal B10 lymphoid cells responding to B10.D2 stimulators, as well as addition of chimeric cells to the reciprocal B10.D2 anti-B10 response.

Specific Tolerance to Allogeneic Donor-type Lymphoid Elements as Evidenced by CML. Cells from the spleens of mixed allogeneic chimeras (B10 + B10.D2 → B10) were examined for in vitro CML sensitization against B10, B10.D2, or

TABLE I

Characterization of Reconstitution of Fully Allogeneic and Mixed Allogeneic Chimeras

Reconstitution	Animal number	B10.D2 cells* at various weeks after reconstitution:				
		1	2	3	5	48
				%		
—	Normal B10	1	0	0	2	0
—	Normal B10.D2	99	100	96	98	99
Fully allogeneic (B10.D2 → B10)	637	69	93	74	78	100
	638	80	100	100	78	100
	639	78	100	71	NT‡	NT
	640	81	100	100	79	92
	641	84	96	100	100	100
Mixed allogeneic (B10 + B10.D2 → B10)	626	68	70	67	88	62
	627	71	72	80	61	84
	628	74	12	11	53	86
	629	68	90	84	82	100
	630	73	62	73	NT	NT

Complement-mediated cytotoxicity was performed on PBL by trypan blue exclusion microcytotoxicity assay.

* Percentage of B10.D2 was calculated by normalizing the total percent specific kill by the two antisera used (anti-H-2^b and anti-H-2^d) to 100%, after correction for percent lysis by complement (C) alone: [(percent B10.D2 − C)/(percent B10.D2 + percent B10) − 2(C)] × 100.

‡ Animal not tested because of death or sacrifice.

TABLE II

Host Hyporeactivity and Third-party Reactivity of Mixed Allogeneically Reconstituted Animals in One-way MLR (B10 + B10.D2 → B10)

Animal	[^{3}H]Thymidine uptake		
	Anti-B10	Anti-B10.D2	Anti-B10.BR
		cpm	
Normal B10	4,877 ± 408	31,584 ± 2,433	34,800 ± 2,921
Normal B10.D2	22,788 ± 888	2,763 ± 267	19,724 ± 729
Chimera A	3,347 ± 237	3,197 ± 435	14,762 ± 303
Chimera B	2,725 ± 292	2,427 ± 456	11,600 ± 250

	Stimulation index	
	Anti-B10.D2	Anti-B10.BR
Normal B10	6.5	7.1
Normal B10.D2	8.3	7.1
Chimera A	0.9	4.3
Chimera B	0.9	4.5

Mean ± SEM of triplicate cultures at 1:1 responder/stimulator ratio. Representative MLR illustrating the responses of two individual animals. There was little animal-to-animal variation.

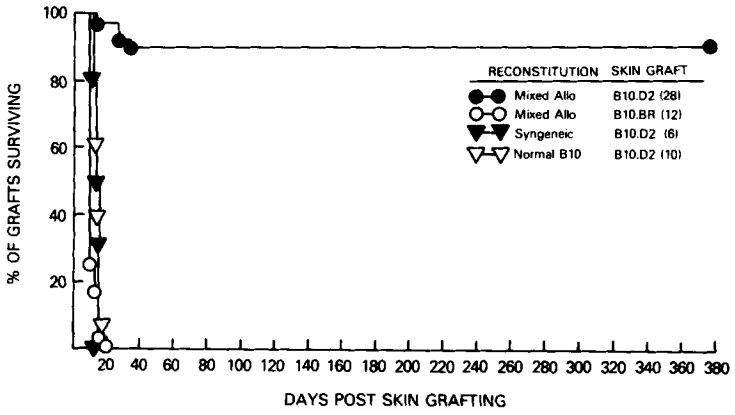

FIGURE 4. Skin allograft survival in mixed allogeneic chimeras, as calculated by the life table method. Skin grafts were followed for a minimum of 94 d.

B10.BR stimulator cells in one-way CML. Corresponding cultures of chimeric responding cells produced no detectable cytotoxic effectors for host (B10)- or donor (B10.D2)-type Con A blast targets, but produced effectors capable of lysing B10.BR third-party blasts with an efficiency similar to that of nonirradiated control B10 and B10.D2 responders. In addition, chimeric lymphoid populations depleted of either H-2^b- or H-2^d-bearing cells were specifically tolerant to host- and donor-type stimulator cells, but normally reactive to third-party cells (Fig. 4). To test for the possibility of active suppression, normal B10 responding cells were cocultured with unirradiated spleen cells from chimeras, and B10.D2 or third-party B10.BR stimulators. In these experiments, no suppression of the B10 anti-B10.D2 response nor the B10 anti-B10.BR response could be detected, offering no evidence for specific or nonspecific suppression.

Specific Tolerance to Allogeneic Donor-strain Skin Grafts in MHC-congenic Mixed Allogeneic Chimeras. Consistent with our previous findings (9), mixed allogeneically reconstituted mice (B10 + B10.D2 → B10) demonstrated specific tolerance to donor-type B10.D2 full-thickness tail skin grafts (MST >380 d) (Fig. 5). Rejection of third-party allografts followed a time course similar to normal B10 mice and syngeneically (B10 → B10) reconstituted recipients (MST = 14 d). All retained grafts were soft and appeared healthy, without evidence of chronic rejection or inflammation, and they persisted in situ without contraction for the life span of the animal. These findings were also extended to another mixed-MHC congenic combination (B10 + B10.BR → B10), which likewise showed mixed chimerism and tolerence (Table III).

Assessment of MHC-noncongenic Mixed Allogeneic Model; Skin Grafting, Chimerism, and Serologic Reactivity. To assess whether such complete acceptance of skin grafts could be achieved in strains differing in both MHC and background, mixed allogeneic chimeras (B10 + C3H/HeJ → B10) were produced, and grafted with allogeneic donor-type (C3H; H-2^k) and third-party (B10.D2; H-2^d) skin grafts. In these animals, third-party skin grafts were rejected with a time course similar to unirradiated controls. The donor-type skin grafts were accepted, and initially appeared soft and healthy; however, after ~30 d the onset of induration was noted, and the grafts then followed a course consistent with prolonged chronic rejection. All grafts eventually contracted in size, and were considered rejected by 90 d (MST = 66 d) (Table III). Sera from two separate bleeds, taken

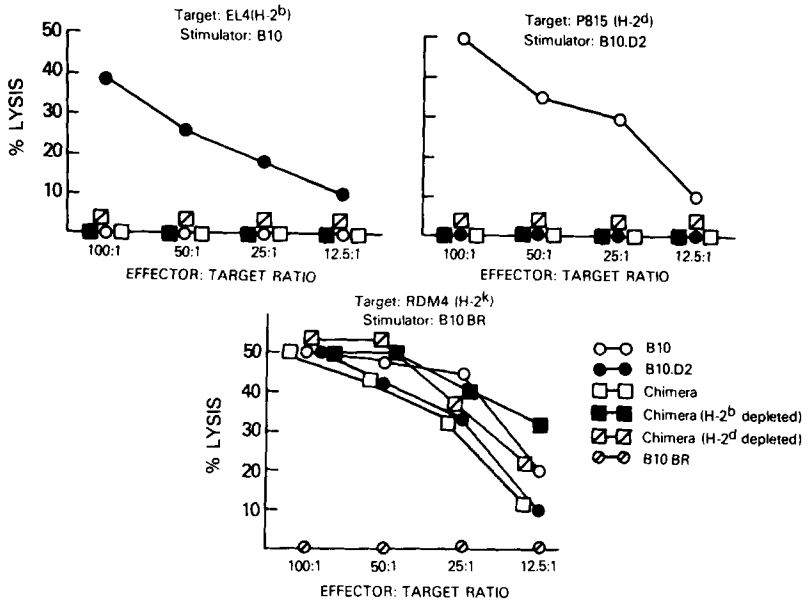

FIGURE 5. Representative experiment of specific cytotoxic T lymphocyte lysis by mixed allogeneic chimeric splenocytes of Cr^{51}-labeled target cells in one way CML to host, donor, and third-party targets. Spontaneous release was <25% unless otherwise indicated. B10 + B10.D2 → B10 spleen cells were untreated (□—□), depleted of H-2^b- (■—■), or H-2^d- (□—□) bearing cells by specific sera and tested in the assays. Efficacy of cellular depletions were confirmed by fluorescence-activated cell sorter using biotinylated protein-purified anti-H-2^b and anti-H-2^d monoclonal antibodies and FITC-avidin fluorescent stain.

TABLE III

Skin Graft Acceptance in MHC-congenic and MHC and Minor Disparate Strains

Reconstitution	Animal number	Skin graft survival			
		Donor strain skin graft	Third party* skin graft	Median survival time	
				Donor	Third party
Mixed allogeneic MHC-congenic (B10 + B10.BR → B10)	1	149‡	14	>149	19
	2	149‡	23		
	3	149‡	19		
Mixed allogeneic MHC-congenic (B10 + B10.D2 → B10)	4	>250	14	>250	19
	5	>250	19		
	6	>250	23		
	7	>250	19		
Mixed allogeneic (B10 + C3H → B10)	8	66	19	66	12.5
	9	66	23		
	10	88	23		
	11	59	14		
	12	90	11		
	13	TF	11		
	14	40	11		
	15	88	11		

* Third party was congenic B10.D2 or B10.BR.

‡ Animal was sacrificed for in vitro cellular assays. Graft survival is recorded as intact up to date of death.

from the animals at the time of most severe graft inflammation and rejection were not cytotoxic for C3H or B10.BR (H-2^k) target spleen cells by trypan blue cytotoxicity assay (data not shown). On day 70, these same animals were grafted with B10.BR skin grafts in order to assess the status of anti-MHC immunity. Such MHC-disparate congenic allografts were accepted, and have remained intact without evidence of acute or chronic inflammation for longer than 100 d.

PBL typing of MHC-noncongenic allogeneic chimeras was carried out by trypan blue microcytotoxicity assay before the onset of chronic rejection of skin allografts, and at the height of the chronic rejection, or after rejection (Table IV). Although the percentages of C3H allogeneic cells detectable fluctuated, all animals remained mixed chimeras despite rejection of C3H skin grafts.

Discussion

Numerous studies (8, 18, 19) have demonstrated that elimination of mature T cells from donor bone marrow inocula can lead to long-term survival of fully allogeneic radiation bone marrow chimeras in specific pathogen-free facilities. However, the survival of such animals is generally not as good as that of syngeneically reconstituted controls, and the health of the animals is often compromised, probably as a result of immunoincompetence due to a failure of appropriate immune cell interactions (6–8). These observations are especially true for animals housed in conventional facilities (6, 7), where survival has been reported to be very poor. In vitro studies of restriction by Singer et al. (8) have

TABLE IV

Characterization of MHC-disparate Congenic and Noncongenic Mixed Allogeneic Chimeras

Reconstitution	Animal number	111 d after reconstitution		141 d after reconstitution	
		H-2^k or H-2^d donor-type cells*	Donor skin graft status‡	H-2^k or H-2^d donor-type cells*	Donor skin graft status‡
		%		%	
—	Normal B10	1		0	
—	Normal C3H	99		99	
Mixed allogeneic MHC-congenic (B10 + B10.BR → B10)	1	5	on	1	on
	2	11	on	73 (very few cells)	on
	3	NT	on	28	on
(B10 + B10.D2 → B10)	4	64	on	25	on
	5	5	on	31	on
	6	74	on	70	on
	7	61	on	38	on
Mixed allogeneic MHC-noncongenic (B10 + C3H → B10)	8	27	on	63	off
	8	23	on	43	off
	10	44	on	42	on
	11	48	on	63	off
	12	85	on	91	on
	13	20	TF§	53	TF
	14	49	on	63	off
	15	44	on	50	on

* Percent H-2^k-bearing lymphoid cells was calculated by normalizing the total percent specific kill by the two antisera used (anti-H-2^b and anti-H-2^k) to 100% after correction for percent lysis by complement (C) alone. [(Percent H-2^k − C)/(% H-2^k + % H-2^b) − 2(C)] × 100. Percent H-2^d-bearing allogeneic donor-type cells was calculated in a similar fashion for animals 4–7.

‡ Animals were grafted 90 d after lethal irradiation and reconstitution with allogeneic donor-strain skin identical to bone marrow transplant.

§ TF, technical failure.

demonstrated that lymphocytes from fully allogeneic chimeras are competent, but are apparently restricted to interacting with accessory cells that express host, but not donor, MHC determinants. In these studies, intact spleen cells from such chimeras were not capable of generating responses to trinitrophenol–keyhole limpet hemocyanin (TNP-KLH) unless accessory cells of host type were added to in vitro cultures, supporting the hypothesis that the immunoincompetence may be due to a failure of appropriate immune cell interactions in these animals.

These limitations may be overcome by reconstituting the murine recipient with a mixture of syngeneic plus allogeneic bone marrow (8, 9). We hypothesize that the allogeneic lymphoid component provides a milieu for induction of specific transplantation tolerance to the donor strain, while the presence of syngeneic lymphoid elements abrogates the limitations of immunoincompetence observed in fully allogeneic chimeras in vivo and in vitro.

The mixed allogeneically reconstituted mice (B10 + B10.D2 → B10) were found to be immunocompetent in B cell and helper T cell function, as assessed by primary PFC responses to in vivo SRBC immunization. Their responses did not differ significantly from those of syngeneically (B10 → B10) reconstituted mice. This was in contrast to fully allogeneic chimeras, the majority of which did not respond. Further evidence for the immunocompetence of such mixed allogeneic chimeras is provided by their superior survival in a conventional animal facility in comparison with fully allogeneic chimeras, the majority of which succumbed to endemic viral infections.

The mixed allogeneically reconstituted mice (B10 + B10.D2 → B10) represent true mixed chimeras, bearing variable percentages of syngeneic and allogeneic lymphoid elements in their peripheral blood. Such chimerism has been maintained for >48 wk after bone marrow transplantation. These findings are in contrast with our model for mixed xenogeneic reconstitution (B10 + F344 rat → B10), where only low levels, if any, of donor-type lymphoid elements could be identified, despite acceptance of donor-type skin xenografts and specific in vitro tolerance to xenogeneic donor strain by MLR and CML (20). In addition, the percentage of allogeneic donor-strain cells present in such mixed allogeneic chimeras did not appear to have an influence on the degree of tolerance to donor strain, as evidenced by specific skin graft acceptance and in vitro responses in CML and MLR.

Despite reconstitution of the host with three times more allogeneic T cell–depleted bone marrow cells than identically treated syngeneic cells in the mixed allogeneic model, the animals manifested a variable range of mixed chimerism. The proportion of allogeneic donor-type (B10.D2) cells ranged from 5% to 95% in individual animals.

In contrast with MHC-congenic mixed allogeneic chimeras, in which the allogeneic (B10.D2, B10.BR) donor-strain skin allografts were specifically accepted indefinitely, without evidence for chronic rejection donor-strain skin allografts from MHC-noncongenic mixed allogeneic chimeras (B10 + C3H → B10) underwent a chronic rejection reaction, with ultimate disappearance of the donor-type (C3H) skin graft. However, allogeneic donor-strain (C3H) lymphoid elements persisted in the hematopoietic system, despite rejection of the C3H skin grafts, and sera taken from the animals during the rejection process were not cytotoxic for C3H splenocytes. In addition, B10.BR skin grafts placed on those animals after rejection of the C3H ($H\text{-}2^k$) grafts were accepted indefinitely, without evidence of inflammation. This disparity of response to hematopoietic elements vs. skin allografts is most likely due to skin-specific alloantigens, perhaps similar to those characterized by Boyse et al. (21) and Steinmuller et al. (22). If so, subsequent survival of B10.BR allografts indicates that such alloantigens must be encoded by genes outside the MHC. The appearance of prolonged chronic rejection with eventual slow contraction of the skin allografts and, ultimately, rejection, is reminiscent of the results we reported (20) in the mixed xenogeneic system (B10 + F344 rat → B10), suggesting that the ultimate rejection of skin grafts in this model may also have been due to skin-specific antigens. Studies are currently in progress to assess the in vitro reactivity of these MHC-noncongenic mixed allogeneic chimeras.

Rejection of noncongenic skin grafts does not necessarily imply a problem for the adaptation of this model to transplantation of vascularized organs, since mismatching for non-MHC antigens does not appear to be of great significance for the outcome of renal allografts in man (23). In addition, skin-specific alloantigens may be among the few minor transplantation antigens not also expressed on bone marrow cells. In this regard, Steinmuller and Lofgreen (22) reported acceptance of cardiac allografts in radiation bone marrow chimeras despite the rejection of donor-type skin grafts. Skin may indeed be the most difficult allograft to prolong, and studies of vascularized grafts in this model are now in progress.

Mixed allogeneic reconstitution may provide a model for investigation of the mechanisms of induction and maintenance of specific tolerance to allogeneic lymphoid elements. In addition, it may offer applications to the clinical bone marrow and solid organ transplantation setting. Conditioning of the host with single high-dose irradiation (or perhaps other suppressive regimens) and immediate mixed allogeneic bone marrow reconstitution coincident with solid organ transplantation could afford immediate use of donor organs with the potential for acceptance of the donor allograft in the absence of prolonged nonspecific chemical immunosuppressive agents. One major advantage of such mixed bone marrow reconstitution would be the ability to overcome the immunoincompetence observed in fully allogeneic chimeras, while inducing and maintaining specific tolerance to the donor. Attempts to extend these studies to a large animal model are now progress.

Summary

Mixed allogeneically reconstituted mice (B10 + B10.D2 → B10) that specifically accept B10.D2 tail skin allografts were examined for in vivo and in vitro immunocompetence, patterns of hematopoietic repopulation, and in vitro reactivity. In vitro, mixed allogeneic chimeras (B10 + B10.D2 → B10) manifested specific tolerance in mixed lymphocyte reactions and cell-mediated lympholysis to B10 and B10.D2 splenocytes, with normal responses to third-party (B10.BR) cells. Such chimeras were immunocompetent in B cell and helper T cell responses, as assessed by their primary plaque forming cell responses to in vivo sheep red blood cell immunization. This is in contrast to fully allogeneic chimeras, which responded less well. In addition, survival of the mixed allogeneic chimeras in a conventional animal facility was superior to that of fully allogeneic chimeras, and similar to syngeneically reconstituted (B10 → B10) mice. Specific tolerance to skin grafts, degree of allogeneic engraftment, and persistence of chimerism was also assessed in a noncongenic mixed allogeneic combination (B10 + C3H → B10). Such animals manifested specific hyporeactivity to C3H skin allografts, but eventual chronic rejection of the grafts occurred in spite of stable and persistent mixed chimerism. MHC-congenic (B10.BR) skin grafts were accepted indefinitely in the same animals, suggesting that skin-specific non–major histocompatibility complex antigens were responsible for rejection of the C3H skin allografts.

We thank Judith Kress and Judith Steckel for manuscript preparation, John C. Hoffman, William Hinkle, and Terrence R. O'Donovan for excellent care of the animals, and John W. Williams and Brian S. Weatherly for technical assistance. We also thank Gene M.

Shearer, and Richard J. Hodes for review of the manuscript, and Alfred Singer for helpful discussions.

Received for publication 28 February 1985.

References

1. Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance to foreign cells. *Nature (Lond.).* 172:603.
2. Rappaport, F. T. 1977. Immunologic tolerance: Irradiation and bone marrow transportation in induction of canine allogeneic unresponsiveness. *Tranplant. Proc.* 9:891.
3. Slavin, S., S. Strober, Z. Fuks, and S. J. Kaplan. 1977. Induction of specific transplantation tolerance using fractionated total lymphoid irradiation in adult mice: Long term survival of allogeneic bone marrow and skin grafts. *J. Exp. Med.* 146:34.
4. Dittmer, J., and M. Bennett. 1978. Successful cardiac allografts in syngeneic radiation chimeras. *Mol. Cell. Biochem.* 21:83.
5. Myburgh, J. A., J. A. Smit, S. Browde, and H. R. H. Hill. 1980. Transplantation tolerance in primates following total lymphoid irradiation and allogeneic bone marrow injection. I. Orthotopic liver allografts. *Transplantation (Baltimore).* 29:401.
6. Zinkernagel, R. M., A. Althage, G. Callahan, and R. M. Welsh. 1980. On the immunoincompetence of H-2 incompatible irradiated bone marrow chimeras. *J. Immunol.* 124:2356.
7. Rayfield, L. S., and L. Brent. 1983. Tolerance, immunocompetence, and secondary disease in fully allogeneic radiation chimeras. *Transplantation (Baltimore).* 36:183.
8. Singer, A., K. S. Hathcock, and R. J. Hodes. 1981. Self recognition in allogeneic radiation bone marrow chimeras: A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T helper cells. *J. Exp. Med.* 153:1286.
9. Ildstad, S. T., and D. H. Sachs. 1984. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of skin allografts or xenografts. *Nature (Lond.).* 307:168.
10. Singer, A., K. S. Hathcock, and R. J. Hodes. 1979. Cellular and genetic control of antibody responses. V. Helper T-cell recognition of H-2 determinants on accessory cells but not B cells. *J. Exp. Med.* 149:1208.
11. Arn, J. S., S. E. Riordan, D. Pearson, and D. H. Sachs. 1982. Strain restricted typing sera (SRTS) for use in monitoring the genetic integrity of congenic strains. *J. Immunol. Meth.* 55:141.
12. Billingham, R. E. 1961. Free skin grafting in mammals. *In* Transplantation of Tissues and Cells. R. E. Billingham and W. K. Silvers, editors. The Wistar Institute Press, Philadelphia. p. 1.
13. Jones, E. C., and P. L. Krohn. 1960. Orthotopic ovarian transplantation in mice. *J. Endocrinol.* 20:135.
14. Schwartz, R. H., C. G. Fathman, and D. H. Sachs. 1976. Inhibition of stimulation in murine mixed lymphocyte cultures with an alloantiserum directed against a shared Ia determinant. *J. Immunol.* 116:929.
15. Epstein, S. L., K. Ozato, and D. H. Sachs. 1980. Blocking of allogeneic cell-mediated lympholysis by monoclonal antibodies to H-2 antigens. *J. Immunol.* 125:129.
16. Sharrow, S. O., B. J. Mathieson, and A. Singer. 1981. Cell surface appearances of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. *J. Immunol.* 126:1327.
17. Hodes, R. J., and A. Singer. 1977. Cellular and genetic control of antibody responses

in vitro. I. Cellular requirements for the generation of genetically controlled primary IgM responses to soluble antigens. *Eur. J. Immunol.* 125:129.

18. Auchincloss, H., and D. H. Sachs. 1983. Mechanism of tolerance in murine radiation bone marrow chimeras. I. Nonspecific suppression of alloreactivity from spleen cells from early but not late chimeras. *Transplantation (Baltimore).* 36:436.
19. Krown, G. E., R. Coico, M. P. Scheid, G. Fernandez, and R. A. Good. 1981. Immune function in fully allogeneic mouse bone marrow chimeras. *Clin. Immunol. Immunopathol.* 19:268.
20. Ildstad, S. T., S. M. Wren, S. O. Sharrow, D. Stephany, and D. H. Sachs. 1984. In vivo and in vitro characterization of specific hyporeactivity to skin xenografts in mixed xenogeneically reconstituted mice (B10 + F344 Rat → B10). *J. Exp. Med.* 160:1820.
21. Boyse, E. A., E. M. Lance, E. A. Carswell, S. Cooper, and L. T. Old. 1970. Rejection of skin allografts by radiation chimeras: Selective gene action in the specification of cell surface structure. *Nature (Lond.).* 227:901.
22. Steinmuller, D., and J. S. Lofgreen. 1974. Differential survival of skin and heart allografts in radiation chimeras provides further evidence of SK histocompatibility antigen. *Nature (Lond.).* 248:796.
23. Bergan, J. J. (1975). The 12th report of the human renal transplant registry. *JAMA (J. Am. Med. Assoc.).* 233:787.